ARTIFICIAL PARTHENOGENESIS IN STARFISH EGGS: PRODUCTION OF PARTHENOGENETIC DEVELOPMENT THROUGH SUPPRESSION OF POLAR BODY FORMATION BY METHYLXANTHINES

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ABSTRACT

Methylxanthines such as caffeine, theophylline, and theobromine at 6 to 10 mM activated eggs of the starfish, *Asterina pectinifera*. Up to one hour of methylxanthine treatment induced parthenogenetic development in more than 80% of the eggs that failed to form the second polar body. Eggs that formed two polar bodies did not cleave. Compared with normally fertilized eggs the first cleavage in parthenogenetically developing eggs was delayed by 2 h in eggs lacking a polar body, and by 3 h in eggs with one (first) polar body. The intervals between successive cleavages were identical to those of normally fertilized eggs.

Examination of the chromosome number revealed that most parthenogenetic embryos develop as tetraploids. The possible significance of centriolar material in initiating parthenogenetic development is suggested.

INTRODUCTION

Since Oscar and Richard Hertwig's early success with artificial parthenogenesis (1887, 1896), sea urchin eggs have been the most common material used for experimentation on parthenogenetic development, while parthenogenesis in other marine ova has received much less attention. This is unfortunate because, among marine eggs, the sea urchin egg is unique in completing its meiosis before spawning.

Marine eggs that are fertilizable before completion of meiosis can undergo parthenogenesis. Thus, Tyler (1931, 1932), and Tyler and Bauer (1937) found that artificial parthenogenesis in *Urechis* is associated with suppression of polar body formation. Morris (1917) and Heilbrunn (1925) found that polar body formation and cleavage are mutually exclusive in artificially activated *Cumingia* eggs. Delage (1901) and Lillie (1908) noted that responsiveness of starfish oocytes to parthenogenetic agents declines as the oocyte goes through the first meiotic division. Apparently the formation of the polar body and parthenogenetic development are mutually exclusive.

Recently we found that caffeine and other methylxanthines are potent parthenogenetic agents in starfish eggs. Since methylxanthines inhibit cell division (Cheney, 1948; Roper, 1977; Stallwood and Davidson, 1977; Beetham and Tolmach, 1982), we used these agents to analyze the possible relationships between inhibition of meiosis and induction of parthenogenesis.

MATERIALS AND METHODS

Starfish, *Asterina pectinifera,* were collected during the breeding season and kept in aquaria with circulating cold (15–18°C) sea water until use. Immature oocytes

free of the follicular envelope were prepared by treating isolated ovaries with Ca-free artificial sea water (CaFSW), as previously reported (Nemoto *et al.,* 1980). After several washings with normal sea water (NSW), we treated the isolated oocytes with 10^{-6} M 1-methyladenine (1-MeAde; Sigma Chemical Co.) in NSW to induce meiosis (Kanatani, 1969). Spermatozoa were obtained by making several cuts in isolated testes.

Methylxanthines such as caffeine (Sigma), theophylline (Wako Pure Chemical Co.), theobromine (Wako), and isobutyl-methylxanthine (Sigma) were dissolved in either NSW or CaFSW. These methylxanthines, at concentrations of 2–14 mM, were applied to eggs at various stages during meiosis. Technical details are described in Results.

To count chromosomes of the parthenogenetically developing embryos, the fertilization membrane was removed by washing with 1 M urea within a few minutes after the fertilization membrane began to elevate (Ikeda *et al.,* 1976). The denuded eggs were then transferred into either NSW or caffeine SW. The caffeine-treated eggs were returned to NSW 50 minutes later and allowed to develop. At the 16–32 cell stage eggs were treated with 3×10^{-4} M colchicine (Wako) in NSW for 10–15 minutes and then induced to swell by transferring them into diluted SW (30%). They were fixed for several hours at room temperature in a mixture of glacial acetic acid and absolute methanol in a 1:3 ratio and stored in 70% ethanol at $-20°$C. They were stained with aceto-orcein (Merck) for several days. Stained blastomeres were squashed on a slide glass and photographed or sketched to count the number of metaphase-arrested chromosomes.

RESULTS

Activation of maturing oocytes by methylxanthines

Caffeine was found to elevate the fertilization membrane in maturing oocytes. Figure 1A shows that 6 mM caffeine is the minimum concentration for formation
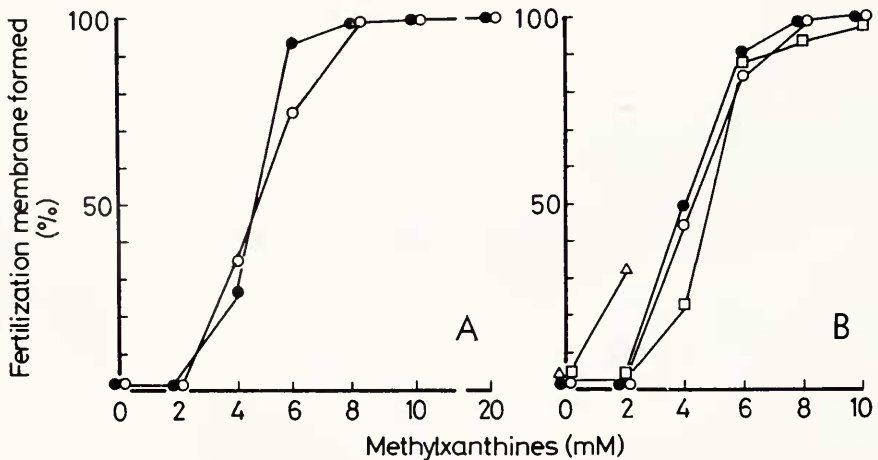

FIGURE 1. Methylxanthine concentrations required for the formation of a fertilization membrane (20°C). Each methylxanthine was applied to eggs between first and second polar body extrusion. (A) Caffeine in NSW (closed circle) and in CaFSW (open circle). Points are averages of 3 experiments. (B) Theophylline in NSW (closed circle) and in CaFSW (open circle); theobromine (square) and isobutyl-methylxanthine (triangle) in NSW. Points are averages of 2 experiments.

of the fertilization membrane in most eggs at any maturation stage after germinal vesicle breakdown (GVBD), just as the oocytes are fertilizable at any stage. The fertilization membrane became visible a few minutes after the application of caffeine, and elevated slowly taking several tens of minutes as in normally fertilized eggs. The formed membrane appears to be intact (Fig. 4A). Concurrently with the elevation of the fertilization membrane, oxygen consumption of the treated eggs increased transiently as in normal fertilization (Nemoto, Washitani, and Hino, in prep.). The term "activation" here means formation of the fertilization membrane by caffeine and other methylxanthines. The formation of the fertilization membrane following 10 mM caffeine treatment was inhibited by 4–6 mM procaine which suppresses the breakdown of the cortical granules of fertilized sea urchin eggs (Vacquier, 1975).

Other methylxanthines, such as theophylline and theobromine, had the same effects (Fig. 1B). Two mM isobutyl-methylxanthine also activated about 30% of the treated eggs. Owing to low solubility in NSW, however, the effects at higher concentrations could not be examined. Asterias amurensis and Astropecten scoparius eggs were also activated by these methylxanthines under similar concentrations (data not shown). Such effects of these methylxanthines were independent of Ca^{2+} in the external medium (Fig. 1A, B).

Duration of treatment with caffeine necessary to induce cleavage

The treatment with methylxanthines for a few minutes activated eggs as seen by elevation of a fertilization membrane; longer treatment allowed eggs to develop further if the eggs were returned to NSW after the treatment (see Figs. 4 and 5). In the experiments shown in Figure 2, caffeine (8 or 10 mM) was applied to eggs between first and second polar body formation. Fifty to ninety minute treatments were optimal
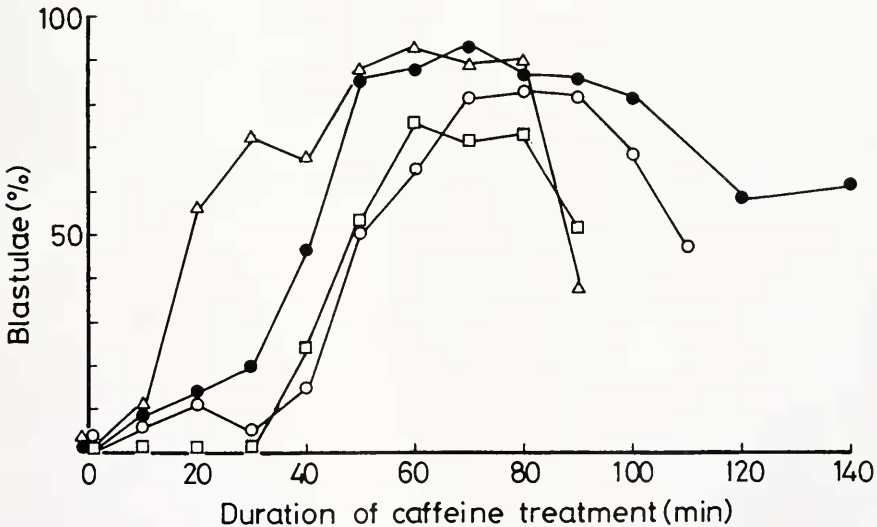

FIGURE 2.   Duration of caffeine treatment required for parthenogenetic development (20°C). Caffeine at 8 mM (open circle) or 10 mM (other symbols) was applied to eggs between the first and second polar body extrusion. The number of developing embryos was scored (>500 eggs) about 10 hours after caffeine treatment. Points are averages for duplicate experiments.

(with some variation among batches) in obtaining a high frequency of blastula formation.

Cleavage and the timing of caffeine treatment during meiosis

The optimum period for insemination resulting in normal cleavage is known to lie between GVBD and the appearance of the first polar body although the starfish egg is fertilizable at any maturation stage after GVBD (Delage, 1901; Lillie, 1915; Fujimori and Hirai, 1979; Kominami and Satoh, 1980). We examined for such an optimum period in methylxanthine treatment by applying caffeine at various stages (Fig. 3A), *i.e.,* (I) within 5 min and (II) 15 min after GVBD, (III) within 5 min and (IV) 15 min after extrusion of the first polar body, and (V) within 5 min and (VI) 20 min after extrusion of the second polar body. The concentration of caffeine and duration of the treatment were 10 mM and 60 minutes respectively (Fig. 3B). Blastulae were produced when caffeine was applied before the appearance of the second polar body (treatments I–IV). High blastulae scores (78–84%) were obtained by treatments II, III, and IV. Conversely, very few eggs (less than 1%) cleaved when caffeine was introduced after extrusion of the second polar body (V, VI). Such a change in rate of development with delay of activation was noted by Delage (1901) in *Asterias glacialis* eggs and Lillie (1908) in *Asterias forbesii* eggs.

The majority of the eggs in treatment III started to divide about 3–4 hours after removal from caffeine SW to NSW (Fig. 4, A–D and Fig. 5, G–N). Once cleaved, the eggs usually continued to cleavage, reaching the blastula stage approximately 15 hours after the treatment and the early gastrula stage in another 20 hours. However, a large number of the eggs in I, V, and VI remained undivided, and eventually disintegrated (Fig. 4E, F).
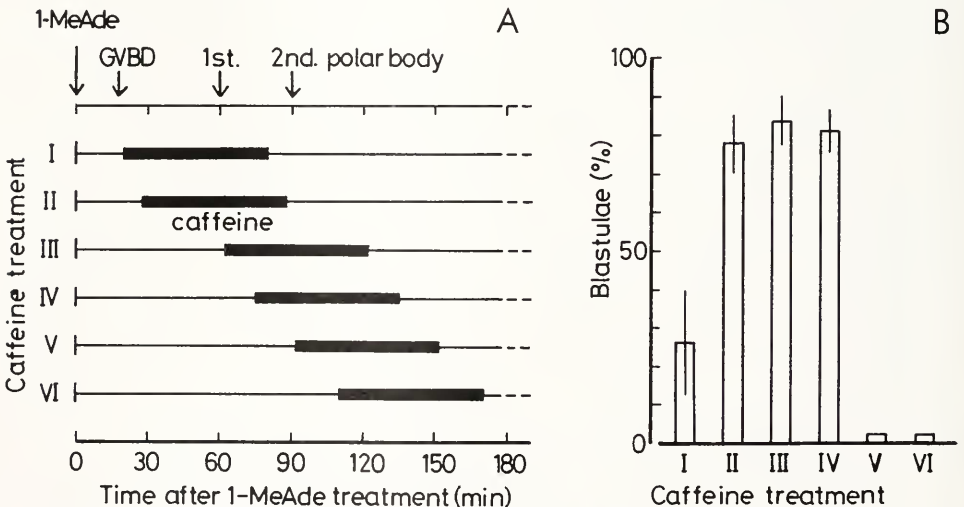

FIGURE. 3. Parthenogenesis of the eggs treated with 10 mM caffeine for 60 min at the various stages during meiosis (I–VI) as described in Results. 22°C. Time (measured after 1-MA) of the onset of GVBD (about 18 min), the first (60 min) and second (90 min) polar body is shown by arrows. Percentage of developing embryos (n > 500 eggs) 10 hours after caffeine treatment. Averages (columns) ±SEM of 4 experiments are shown.
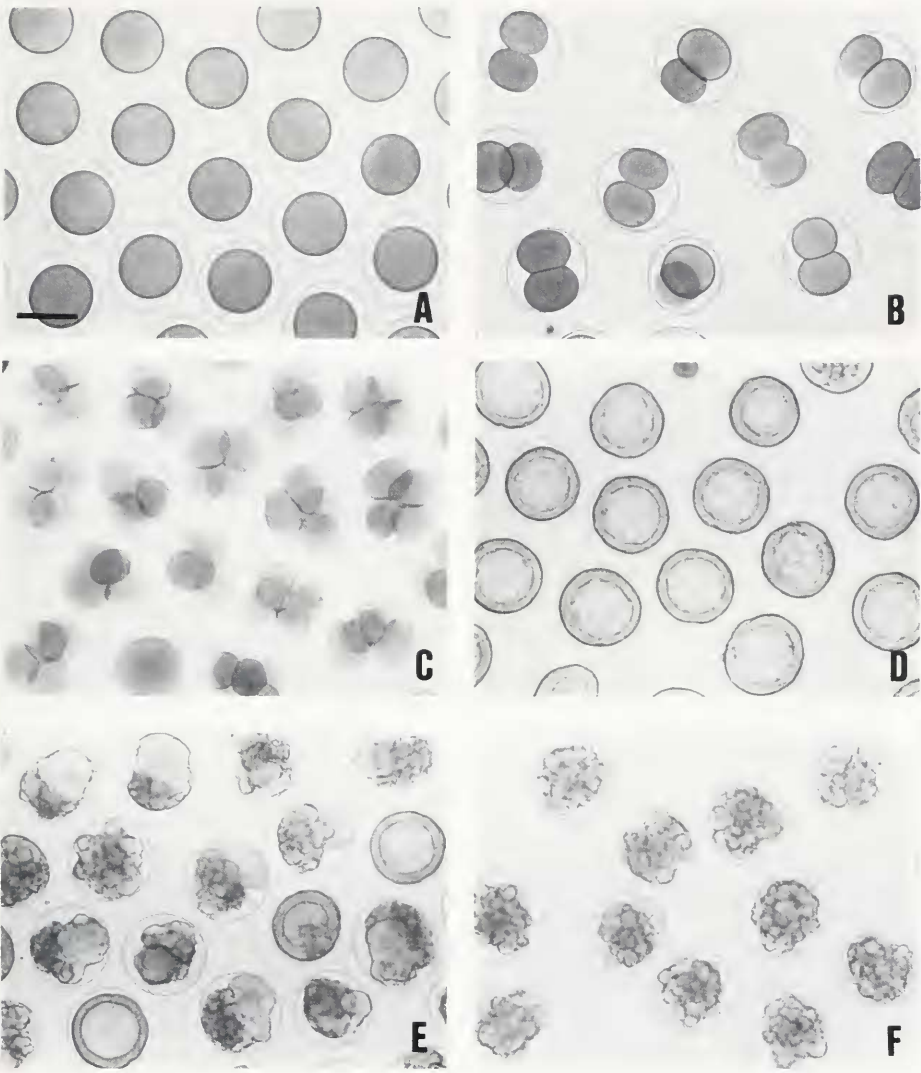
FIGURE 4. Development of eggs treated with caffeine (22°C). A–D: Eggs treated with caffeine shortly after extrusion of the first polar body; (A) 30 minutes after caffeine treatment, (B) 3.5 hours, (C) 4 hours, and (D) 16 hours. E–F: Disintegration of uncleaved eggs about 15 hours after caffeine treatment; (E) eggs treated just after GVBD (treatment I); and (F) eggs treated after extrusion of the second polar body (treatment V). Bar in A representing 100 μm is common to A through F.

While inducing parthenogenetic development, caffeine inhibited the formation of polar bodies in the treated eggs; in treatments III and IV, most eggs failed to extrude the second polar body. When subjected to treatment II, 90% of the eggs did not form any polar bodies. The remaining eggs formed only one polar body after they were returned to NSW, with a considerable delay in time. Eggs treated with caffeine in an earlier stage (treatment I) showed some variation (0–2) in the number

of polar bodies formed. Moreover, two types of abnormal eggs were sometimes found; namely eggs with three polar bodies, and others with a single, distinctly large-sized polar body (Fig. 5, E–F) measuring 11.1 ± 0.6 μm (n = 16) in diameter. The first polar body in control eggs measured 8.2 ± 1.8 μm, n = 16. As in treatment II, polar body formation occurred after the treated eggs had been returned to NSW.

Caffeine and other methylxanthines used here inhibit cytokinesis in a variety of cells (Cheney, 1948; Roper, 1977; Stallwood and Davidson, 1977; Beetham and Tolmach, 1982). The present results show that caffeine also inhibits the meiotic division of starfish oocytes. Delage (1901) also reported that some parthenogenetic agents suppress polar body formation in *Asterias glacialis* oocytes.
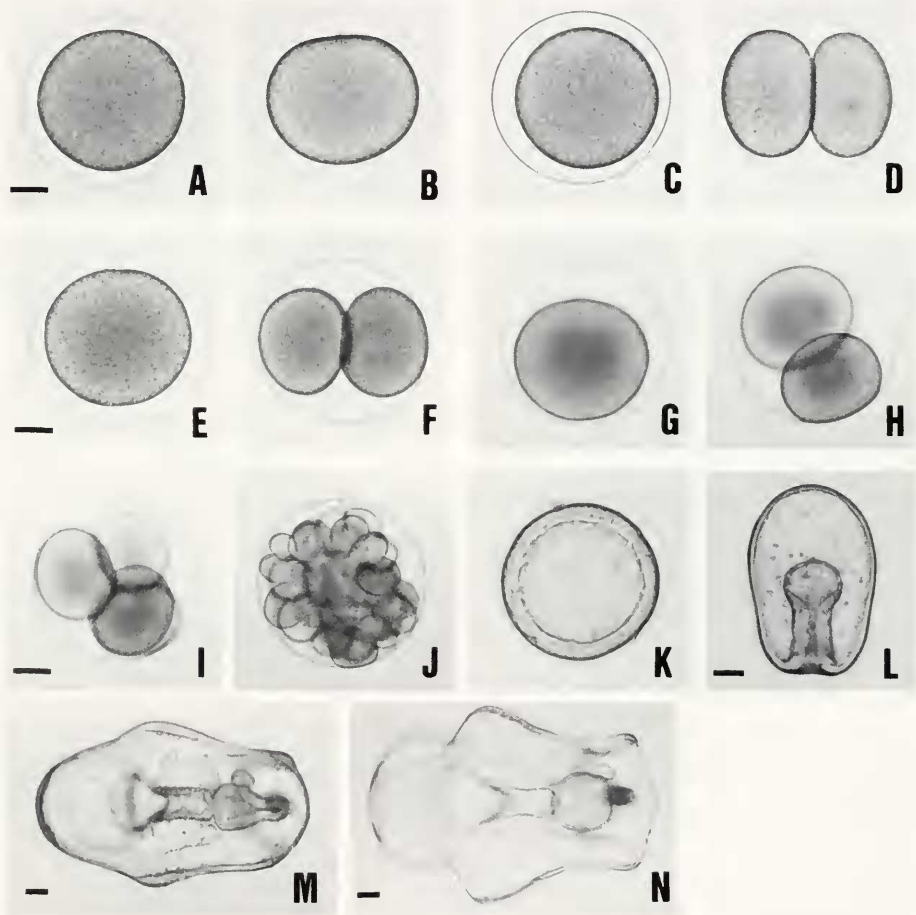

FIGURE 5.   Cleavage of caffeine-treated eggs (22°C). A–B: Control eggs inseminated before first polar body extrusion; (A) 70 minutes and (B) 90 minutes after 1-MeAde treatment. C–D: Eggs with no polar body obtained by treatment II. E–F: Eggs with a giant polar body obtained by treatment I. G–N: Development of eggs with one polar body of normal size obtained from treatment III; (G) 2.5 hours after caffeine treatment (50 min), (H) 3.5 hours, (I) 4.0 hours, (J) 5.5 hours, (K) 16 hours, (L) 30 hours, (M) 3 days, and (N) 5 days. Bars represent 50 μm, and the scale in A is the same for B through K.

From the above it is clear that caffeine treatment can produce five kinds of eggs with respect to the number and size of the polar bodies: none, one, one giant, two, and three polar bodies. The results also suggest a correlation between capability of development and the number of polar bodies formed. To confirm this correlation, batches of eggs treated with caffeine at various meiotic stages were pooled and divided into the above 5 groups by inspecting individual eggs. Each egg was rolled around so that the polar bodies were not overlooked. The number of developing embryos was scored six hours later, and the number of polar bodies was again checked (Table I).

The eggs with either one normal-sized or no polar bodies divided with high frequency, whereas few eggs with either two or three polar bodies divided. The cleavage frequency among eggs with a giant polar body was about half that of eggs with a normal-sized polar body.

Caffeine as a parthenogenetic agent was found to induce, in some eggs, the formation of a giant polar body which was 2.4 times $(=[11.1/8.2]^3)$ as large in volume as that of the controls. The appearance of such giant polar bodies also has been reported in artificially activated *Cumingia* (Morris, 1917; Heilbrunn, 1925) and *Urechis* eggs (Tyler, 1931). It is still unknown whether the formation of the giant polar body is causally related to parthenogenetic activation.

Cleavage timing

At the first cleavage, most caffeine-treated eggs divided into two cells (Fig. 5), although some eggs divided into three or four. Subsequent cleavages proceeded synchronously, and the cleaving eggs developed at least to the gastrula stage (Fig. 5, G–N).

After fertilization or caffeine treatment, individual eggs were transferred into separate wells of a culture plate. Direct observations were made every 5 min. An example of 5 experiments is shown in Table II. The first cleavage of caffeine-treated eggs was delayed as compared with that of control eggs. The time delay was about two hours in activated eggs with no polar body and about three hours in eggs with one normal-sized polar body. Hatching was also delayed corresponding to the delay of the first cleavage.

Synchronous cleavages of caffeine-treated eggs took place at 30 min intervals, which was nearly the same as that of control fertilized eggs. The time taken for hatching after the first cleavage did not differ significantly from that of the controls.

TABLE I

Number of polar bodies and rate of cleavage

Number of polar bodies formed	Eggs examined	Cleaved	Cleavage (%)
0	310	272	87
1 (normal)	356	317	89
1 (giant)	173	68	39
2	167	14	8
3	3	0	0

Five batches of eggs were used. Eggs of each batch were divded into four groups of caffeine treatments; I, II, III, and V (see Results). Five kinds of caffeine-treated eggs were selected before the first cleavage, and the rate of cleavage was counted 5–6 hours after caffeine treatment (10 mM and 60 min).

TABLE II

Timing of cleavages and hatching

	Time after 1-MeAde treatment (22°C)					
	1st	2nd	3rd	4th	5th	Hatch
(1)	4:00	4:20	4:50	5:40	6:20	16:00
Caffeine-treated eggs	4:15	4:45	5:15	5:40	6:00	16:35
with no polar bodies	4:05	4:35	5:05	5:30	5:55	15:40
	4:20	4:45	5:15	5:40	6:20	15:45
	4:05	4:30	5:05	5:25	6:00	16:10
	4:10	4:40	5:15	5:40	6:00	16:00
	4:10	4:40	5:15	5:40	6:05	15:30
	4:05	4:30	5:05	5:30	5:55	15:30
Mean	4:08	4:36	5:08	5:36	6:04	15:53
Interval		0:28	0:32	0:28	0:28	11:45*
(2)	5:10	5:20	6:05	6:30	7:05	17:05
Caffeine-treated eggs	5:20	5:45	6:05	6:30	7:15	17:15
with one polar body	5:15	5:45	6:10	6:35	7:05	16:45
(normal size)	5:05	5:30	6:05	6:30	7:05	16:55
	4:50	5:30	6:05	6:30	6:55	17:10
	5:30	5:55	6:20	6:45	7:15	17:35
	4:40	5:15	5:45	6:20	6:45	17:40
Mean	5:07	5:36	6:05	6:31	7:05	17:13
Interval		0:29	0:29	0:26	0:34	12:06*
(3)						
Control eggs						
Mean (n = 16)	2:14	2:42	3:10	3:38	4:10	13:59
Interval		0:28	0:32	0:28	0:28	11:45*

* Interval (hours and minutes) between the first cleavage and hatching.
Eggs were treated with caffeine (10 mM and 60 min) (1) after GVBD (treatment II) and (2) after extrusion of the first polar body (treatment IV). Control eggs were fertilized before extrusion of the first polar body.

Prior to the first cleavage, multiple nuclei appeared in the caffeine-treated eggs. Subsequently they fused, and then disappeared to enter mitosis. In *A. glacialis*, Tsukahara and Ishikawa (1980) have described the appearance of multiple nuclei when polar body formation was suppressed by cytochalasin B, and their fusion after thymol activation.

Number of chromosomes in normal and parthenogenetic embryos

Makino and Niiyama (1947), counted 40 chromosomes (diploid) in sectioned preparations of male *A. pectinifera* gonads. In our experiments, 44 (2n) chromosomes were found in normal embryos, although a slight variation was noticed (Fig. 6). In caffeine-induced embryos the number of chromosomes ranged from 78 to 107, with a median of 88 (Fig. 7). No significant difference in chromosome number was found
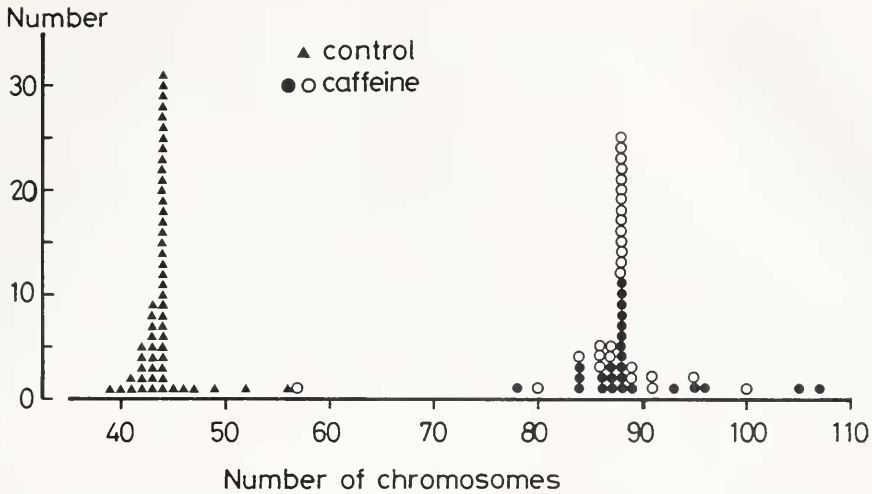
FIGURE 6. Histogram of chromosome number. Most control blastomeres had 44 chromosomes (triangle). A peak of chromosome number in caffeine-induced embryos with one polar body (open circle) and with no polar bodies (closed circle) was 88. Mosaic of chromosome numbers in individual parthenogenetic embryos was not found.

between parthenogenetic embryos with no polar body (closed circle) and one polar body of normal size (open circle). Obviously parthenogenetic embryos are tetraploids.

Since the intervals between successive cleavages are identical to those of normal eggs as described above, these caffeine-induced embryos must have become tetraploids before the first cleavage. The tetraploid status is maintained at least up to the early gastrula stage (Mita and Obata, unpub. data).

FIGURE 7. Chromosomes of a normal embryo (A) and a caffeine-produced embryo (B). Bar: 10 μm. Chromosome numbers are 44 (2n) and 88 (4n) respectively.

DISCUSSION

The action of methylxanthines on starfish eggs as a parthenogenetic agent may occur in two steps, *i.e.,* the activation of the eggs and the initiation of parthenogenetic development.

An increase in intracellular free calcium is an immediate consequence of fertilization or activation in sea urchin eggs, and sea urchin eggs are activated by those agents that increase intracellular Ca^{+2} (Nakamura and Yasumasu, 1974; Steinhardt *et al.,* 1974, 1977; Vacquier, 1975; Zucker *et al.,* 1978; Hamaguchi and Hiramoto, 1981). Caffeine reportedly releases Ca^{2+} from the sarcoplasmic reticulum of muscle tissues (Weber and Herz, 1968; Endo *et al.,* 1970). Caffeine and the other methylxanthines used here, like Ca^{2+}-ionophores, shorten the length of the "1-MeAde-requiring period" in meiosis reinitiation of starfish oocytes (Nemoto, 1982). Therefore, methylxanthines may cause activation by an increase in intracellular Ca^{2+}. Since these methylxanthines acted independently of calcium in the external medium, Ca^{+2} may be used from some intracellular storage.

Suppression of polar body formation by methylxanthines may be due to a disruptive effect on the meiotic spindle. Harris (1983) recently observed that caffeine prevents normal separation and function of the mitotic centers in fertilized sea urchin eggs, resulting in periodic monoaster formation rather than division. As a result, caffeine inhibits separation of daughter nuclei. In fact we found that the multiple nuclei formed by caffeine treatment subsequently fused.

How such a change in nuclear behavior produces parthenogenetic development is unclear. Possibly the duplication of chromosomes by the fusion of once separated daughter nuclei triggers parthenogenetic development. However, this possibility does not explain the observed chromosome number of parthenogenetic embryos; the fused nuclei of the egg without forming the polar body would be tetraploid, whereas the nuclei of those eggs that formed one polar body would be diploid. The observed embryos derived from both types of eggs all develop as tetraploids. Since the interval between the first and fifth cleavage in caffeine-treated eggs was almost the same as that of the controls, these caffeine-induced embryos must have become tetraploid before the first cleavage. An extra delay of the first cleavage by one hour in eggs with one polar body (Table II) may be the time taken for an extra round of DNA synthesis. Von Ledebur-Villiger (1972) found that the majority of sea urchin embryos developing parthenogenetically from ripe haploid eggs are still tetraploids. How most parthenogenetic embryos develop as tetraploids is still unknown.

The role of centrioles has been emphasized in the development of sea urchin eggs. It is believed that in normally fertilized sea urchin eggs centrioles derived from the sperm act as the mitotic organizing center of the zygote nucleus, since ripe unfertilized eggs lack centrioles (Sachs and Anderson, 1970). Therefore, artificial parthenogenesis in sea urchin eggs has been assumed to involve "*de novo*" formation of centrioles (Sachs and Anderson, 1970; Kuriyama and Borisy, 1983). However, it should be recalled that the actual number of developing eggs is unusually low; only 1–3% (rarely up to 30–40%) of artificially activated eggs develop into blastulae or plutei (Von Ledebur-Villiger, 1972). By contrast, in the present study (*cf.* Fig. 3 and Table I) more than 85% of the starfish oocytes developed to blastulae if treated before formation of the second polar body, whereas only a few percent of mature eggs (two polar bodies) were induced to develop. Since the maturing oocytes should retain at least one set of centrioles for organizing centers of meiotic division, it is possible that the centrioles can be diverted into an organizing center of mitotic division when the

oocytes are activated by caffeine. This would explain the observed high frequency of parthenogenetic development of maturing oocytes in contrast to the very low rate in mature eggs of starfish and sea urchins.

ACKNOWLEDGMENTS

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